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Species-specific phytoplankton growth rates via diel DNA synthesis cycles. III. Accuracy of growth rate measurement in the dinoflagellate *Prorocentrum minimum*

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ABSTRACT: The accuracy of species-specific phytoplankton growth rates estimated by cell cycle analysis was tested with the dinoflagellate *Prorocentrum minimum* (Pav.) Sch. under conditions of altered nitrogen and phosphorus availability. Reduced nutrient availability caused major changes in the duration of cell cycle phases. At the nutrient level of complete f/2 media, the length of the combination of S, G2, and M phases was about 8 h at growth rates of 0.53 to 0.56 d^{-1}. A decrease in PO_4^{3-} or NO_3^- concentration extended the S+G2+M phase to about 15.5 to 17.7 h at growth rates ranging from 0.41 to 0.30 d^{-1}. Changes in phase durations did not significantly affect growth rate estimates. In addition, a minimum growth rate, calculated from the maximum values on phase fraction curves, was shown to be usable as an error detector in some cases. Results support the validity of cell cycle analysis to measure in situ growth rates.

INTRODUCTION

It is desirable to measure the growth rates of individual species of phytoplankton to understand how the environment affects a population. The concept of the cell cycle has been used to measure species-specific growth rates based on the fact that, for unicellular organisms, completion of the cycle directly causes population growth (Weiler & Chisholm 1976, McDuff & Chisholm 1982, Carpenter & Campbell 1988). The technique, as typically employed, requires 2 pieces of information to calculate a growth rate (McDuff & Chisholm 1982). One is the daily averaged fraction of a population in a 'terminal event' (Fig. 1), such as mitosis. The other is the duration of that event. In general, the latter is not readily obtainable, as the duration of all cell cycle phases varies according to species and growth conditions (Olson et al. 1986).

Two approaches have been developed to deal with the difficulty of estimating growth rates by the cell cycle analysis method. In one case either the duration of the terminal event is long or the degree of synchrony is high, so that during a particular interval all cells which will divide in a 24 h cycle are in the terminal event. By plotting fractions of cells in the terminal event against time, a plateau is observed on the resultant phase fraction curve, and the duration term may be ignored in the calculation of growth rate (μ) (McDuff & Chisholm 1982). The relationship between growth rate and the fraction indicated by the plateau (f_{max}) is

$$\mu = \ln (1+f_{max}) \quad (1)$$

If not all proliferating cells are present in the terminal event simultaneously, then Eq. (1) always underestimates the true μ, i.e.

$$\mu_{min} = \ln (1+f_{max}) < \mu \quad (2)$$

The other approach involves monitoring simultaneously the fractions of 2 adjacent stages in a terminal event. For example, the double nucleated stage and cytokinesis are 2 such stages used when the M phase is a terminal event (Weiler & Chisholm 1976). In a par-

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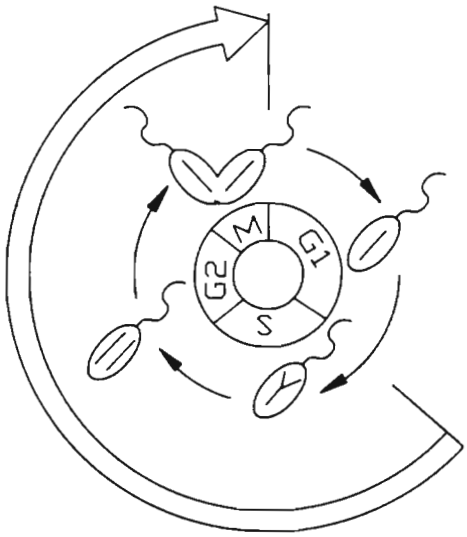

Fig. 1. Eukaryotic cell cycle and its component phases. G1 and G2 are 2 gap phases with respect to DNA synthesis. In the S phase a cell duplicates its DNA (shown as a line within the cells); and mitosis is performed in the M phase. A terminal event is defined as the period between any point on the cell cycle and the end of mitosis (Mitchison 1971). Thick arrow indicates the terminal event used in this study, that is, the combination of S, G2, and M phases.

tially synchronized population, the time lag between analogous features in these fraction curves can be used to deduce the duration of the terminal event. The method derived by Carpenter & Chang (1988) based on the same concept has been tested for both precision and accuracy by measuring growth rates of the dinoflagellate *Heterocapsa triquetra* under nutrient replete conditions (Chang & Carpenter 1988). However, the validity of the method has not been tested under conditions of altered nutrient availability.

This study reports on testing of both approaches of cell cycle analysis using another dinoflagellate, *Prorocentrum minimum* (Pav.) Sch. with alterations in the availability of both nitrogen and phosphorus. In these experiments, the intention was not to reduce the concentrations of N and P to limiting values, but they were altered sufficiently to induce changes in cell cycle phases. Therefore, we refer to these cells with decreased N or P availability as being grown under 'reduced' rather than 'limiting' nutrient conditions.

MATERIALS AND METHODS

An isolate of the dinoflagellate *Prorocentrum minimum*, clone 1PM, (Provasoli-Guillard Culture Collection, Bigelow Laboratory, West Boothbay Harbor, ME, USA) was cultured in autoclaved f/2 media (Guillard & Ryther 1962) with no added silicate. In all experiments, artificial seawater was used to prepare the media (Goldman & McCarthy 1978). Batch cultures (2 or 3 l) were grown at 20°C and supplied with 187 μE m^{-2} s^{-1} (measured with a 4π sensor, Biospherical Instruments, CA, USA) of cool white fluorescent light on a 16:8 h L:D cycle. The beginning of the light period was 07:00 h, and onset of dark was at 23:00 h EDT. The environmental time system was employed to record time in this study, with the onset of the light period as Hour 0 (Edmunds & Laval-Martin 1984). The medium for the reduced N treatment had an initial NO_3^- concentration of 331 µM at an N:P ratio of 9:1; the decreased P experiment had 9.0 µM PO_4^{3-} with an N:P ratio of 100:1. Concentrations of N and P were selected on the basis of preliminary experiments (Antia 1987). To test organic P effects, sodium glycerophosphate was used in place of NaH_2PO_4 at the same concentration as in the f/2 medium, i.e. 36.3 µM.

Cell counts were made daily using a Sedgwick-Rafter counting chamber (Guillard 1973). When the culture was in exponential growth, samples were taken for 24 h at 2 h intervals. For all experiments, each sampling period was the fourth day in the exponential phase, and was at least 2 d before the stationary phase. After a 10 min centrifugation at 134 × *g*, each 30 ml sample was immediately preserved in methanol and stored at −15°C. The staining protocols and the procedures employed to quantify single cell DNA content were the same as described previously (Chang & Carpenter 1988). The final concentration of DAPI (4'6-diamindino-2-phenylindole) in the staining solution was 3.3 µg ml^{-1}. Between 350 and 400 cells were measured in each sample to construct a DNA histogram.

The G1, S, and G2+M phase fractions, which will be denoted by f_{G1}, f_S, and f_{G2M}, respectively, were extracted according to the method developed by Fried (1976). Subsequently, the duration of the S+G2+M phase was obtained by (Carpenter & Chang 1988):

$$T_S + T_{G2M} = 2(t_2 - t_1) \quad (3)$$

where T_S = duration of the S phase; T_{G2M} = duration of the G2+M phase; t_1 = time when the cell number curve of S phase reaches its maximum value; t_2 = time when the cell number curve of G2+M phase reaches its maximum value. For each experiment, t_1 and t_2 were estimated by (Chang & Carpenter 1988): (1) selecting maximum values on f_S and f_{G2M} curves directly; (2) fitting polynomial curves to the sporadic phase fractions followed by calculating t_1 and t_2 from the fitted S and G2+M fraction curves; (3) fitting polynomial curves and applying the iterative method of Carpenter & Chang (1988). The daily mean population growth rate, μ, was obtained from (McDuff & Chisholm 1982, Carpenter & Chang 1988):

$$\mu = \frac{1}{(T_S + T_{G2M})n} \sum_{j=1}^{n} \ln [1 + f_S(t_j) + f_{G2M}(t_j)] \quad (4)$$

where n = number of samples obtained in a 24 h cycle; t_j = time of obtaining the j^{th} sample.

The S and the G2+M durations were calculated according to (Carpenter & Chang 1988):

$$T_S = \frac{2(t_2 - t_1)\beta}{\beta + 1}; \; T_{G2M} = \frac{2(t_2 - t_1)}{\beta + 1} \quad (5)$$

where

$$\beta = \frac{\Sigma \ln [1 + f_S(t_j) + f_{G2M}(t_j)]}{\Sigma \ln [1 + f_{G2M}(t_j)]} - 1$$

To estimate μ_{min}, the S+G2+M phase was defined as the the terminal event (Fig. 1). The maximum value of (f_S+f_{G2M}) should thus be used as the f_{max} in Eq.(2). This is equivalent to finding the minimum value on $f_{G1}(t)$ and calculating μ_{min} by:

$$\mu_{min} = \ln [2 - \min (f_{G1}(t))] \quad (6)$$

where $f_{G1}(t)$ = the fitted polynomial curve for G1 phase fractions, and t belongs to the sampling period.

RESULTS

DNA synthesis in *Prorocentrum minimum* proceeded in phase with the 16:8 h L:D cycle. In all the experiments, peaks on the G2+M phase fraction curve occurred between 0 and 2 h after the dark-light transition point (Figs. 2 to 5). The positions of peaks on f_S, however, varied with nutrient availability in the media. In the nutrient replete (f/2 and organic P) cultures, maximum values in the S fraction were observed between Hours 20 and 22 (Figs. 2 and 3). In the cultures with reduced N or P concentration, peaks of f_S appeared between Hours 16 and 18 (Fig. 4 and 5).

For cells grown in complete f/2 medium, the S phase fraction reached a maximum at Hour 22 (Fig. 2). Since the peak on the G2+M fraction curve was observed at Hour 2, the trend of the S fraction curve near Hour 24 implies that the next G2+M peak should appear at the unsampled Hour 26. Based on this deduction, a growth rate of 0.63 d^{-1} was obtained. This value is slightly higher than the growth rate estimated from cell counts (Table 1). The effort of locating t_1 and t_2 with polynomial regression failed because neither the S nor the G2+M fraction curve possessed a local maximum. Both the calculated T_S and T_{G2M} were around 4 h (Table 2).

Substitution of inorganic phosphate with glycerophosphate did not change the growth rate substantially (Table 1). However, organic phosphate caused a major change in the cell cycle (Fig. 3). As indicated by the phase fraction curves, S cells were observed only from Hours 18 to 24. The G2+M phase still formed a peak

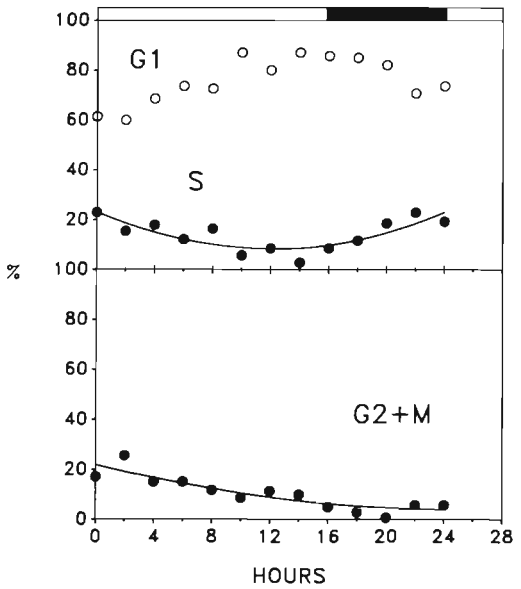

Fig. 2. *Prorocentrum minimum*. Phase fractions for population grown in complete f/2 medium. Initial nutrient concentrations: NO_3^- at 883 µM; PO_4^{3-} at 36.3 µM. Upper panel: (○) G1 phase fractions, and (●) S phase fractions at different times. Lower panel: (●) G2+M phase fractions. Curves are fitted polynomials. Dark bar at top: dark period during 24 h cycle.

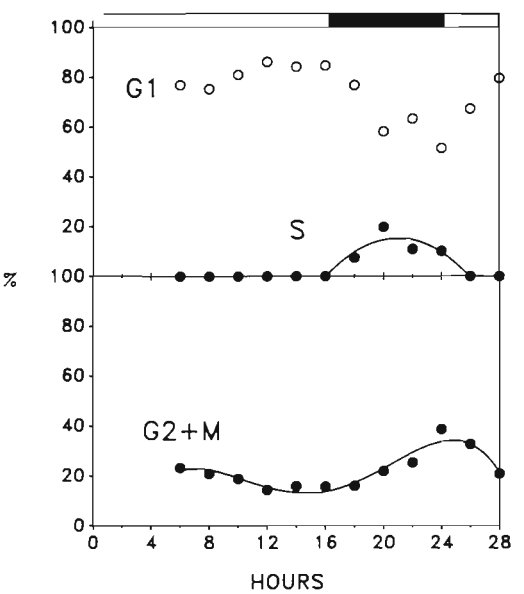

Fig. 3. *Prorocentrum minimum*. Phase fractions for population grown in medium with organic phosphate. Nutrient concentrations: NO_3^- at 883 µM; glycerophosphate at 36.3 µM. Symbols as in Fig 2.

near the dark-light transition point, but the G2+M phase fractions in general exceeded those from the culture with complete f/2 media (Fig. 2). The growth rate estimated by cell cycle analysis was 24% higher

than the μ from cell counts. Compared to complete f/2 culture media, the duration of the S phase was reduced by 75% to only 1.1 h (Table 2). However, the T_{G2M} almost doubled to become 7.1 h.

In contrast to P-replete and organic P experiments, when the availability of orthophosphate was reduced, there was a marked increase in length of the S phase to 10.3 h from the 4.4 h observed in nutrient replete conditions (Table 2; Fig. 4). Before Hour 10, most cells in the population remained in the G1 phase (Fig. 6). However, the DNA histograms between Hours 12 and 22 were dominated by a cell cohort in the S phase. Without curve fitting, the growth rate estimated with DNA analysis was 76% higher than the μ from cell counts. The polynomial regression combined with iteration reduced this difference down to 20% (Table 1).

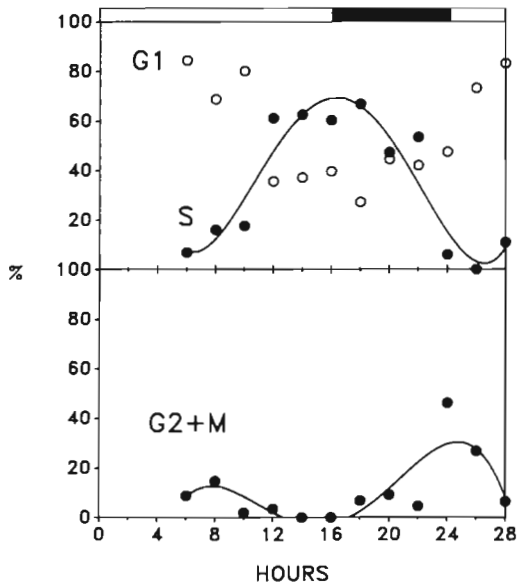

Fig. 4. *Prorocentrum minimum*. Phase fractions for population grown in medium with reduced phosphate concentration. Nutrient concentrations: NO_3^- at 883 µM; PO_4^{3-} at 9.0 µM. Symbols as in Fig. 2

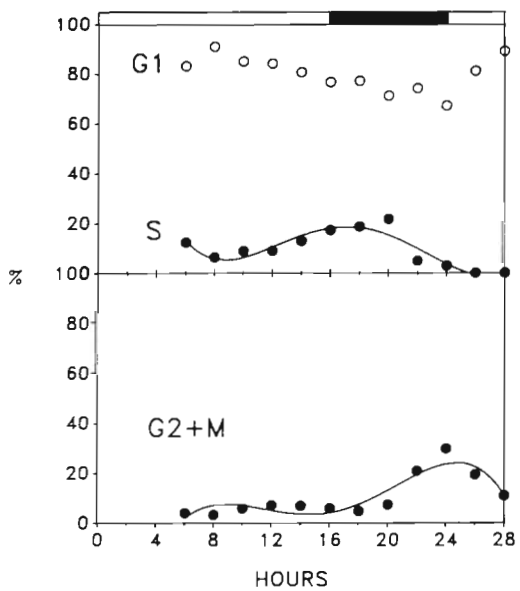

Fig. 5. *Prorocentrum minimum*. Phase fractions for population grown in medium with reduced nitrate concentration. Nutrient concentrations: NO_3^- at 331 µM; PO_4^{3-} at 36.3 µM. Symbols as in Fig. 2

When the nitrogen concentration was reduced, the growth rate estimated via cell counts decreased from 0.56 d^{-1} in the nitrogen replete medium to 0.30 d^{-1} (Table 1; Figs. 2 and 5). An expansion in the duration of both the S and the G2+M phases over nutrient replete values also occurred (Table 2). These changes in length of cell cycle phases and in growth rates did not have a major effect on the estimation of growth rate. In nitrogen-replete media there was an overestimate of the actual growth rate of 13%. In the low nitrogen medium the underestimation of the actual growth rate associated with the curve fitting method was about 7% (Table 1).

All minimum growth rates estimated by f_{max} were lower than μ's estimated with the polynomial regression procedure (Table 1). Most of the μ_{min}'s were also lower than growth rates derived from cell counts. The only exception was the μ_{min} of the culture with reduced phosphate availability. In this particular case, the μ_{min} was 22% higher than μ estimated via cell counts.

DISCUSSION

Both the quantity and the quality of nutrients changed the duration of cell cycle phases in *Prorocentrum minimum* (Table 2). These results are in accordance with previous nitrogenous nutrient experiments on 2 phytoplankton species, *Hymenomonas carterae* and *Thalassiosira weissflogii* (Olson et al. 1986), as well as on the yeast *Saccharomyces cerevisiae* (Rivin & Fangman 1980). On the other hand, the timing of mitosis in *P. minimum* was not altered by the manipulation of nutrients. In all 4 experiments, as indicated by the decrease of f_{G2M}, the onset of mitosis always occurred 2 to 4 h after the dark-light transition point (Figs. 2 to 5). Apparently, the light-dark cycle has a strong control over the phasing of the cell cycle in dinoflagellates (Olson & Chisholm 1983).

When polynomial regression was employed to smooth phase fraction curves, the cell cycle method of measuring species-specific growth yielded values that were similar to growth rates measured via cell counts (Table 1). The mathematical model used to determine growth rate from cell cycle phase fractions and phase

Table 1. *Prorocentrum minimum*. Growth rates (μ; d^{-1}) as estimated by cell counts and cell cycle analysis. Minimum growth rates (μ_{min}) calculated from f_{max} are also listed

Growth conditions	From cell counts	From cell cycle analysis			μ_{min}
		Without curve fitting	Polynomial with iteration	Polynomial without iteration	
Complete f/2	0.56	0.63	–[a]	–[a]	0.36
Organic P	0.53	0.68	0.74	0.66	0.34
Reduced [P]	0.41	0.72	0.52	0.49	0.50
Reduced [N]	0.30	0.54	0.29	0.28	0.25

[a] Growth rate cannot be computed via polynomial regression because there exists no local maximum on regression curves

Table 2. *Prorocentrum minimum*. Duration S and G2+M phases (h) obtained by polynomial regression plus the iterative method

Growth conditions	T_S	T_{G2M}
Complete f/2[a]	4.4	3.6
Organic P	1.1	7.1
Reduced [P]	13.0	4.7
Reduced [N]	7.2	8.3

[a] Durations were calculated without curve fitting

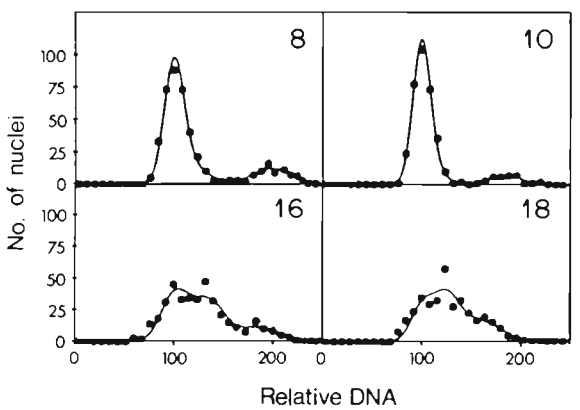

Fig. 6. *Prorocentrum minimum*. Selected DNA histograms from experiment with reduced phosphate concentration. Number in each panel indicates how many hours after the onset of the light period on Day 5 that sample was taken. Points (•) were measured by microfluorometry; curves were fitted by deconvolution procedures (Fried 1976)

duration (Carpenter & Chang 1988) was able to calculate reasonable growth rates even though there were large variations in phase durations. Conversely, when t_1 and t_2 were estimated directly by the location of maximum values of sporadic phase fractions, large error occurred in experiments with reduced nutrient concentrations (Table 1). Baisch et al. (1982) observed that random error involved in the direct extraction of phase fractions occasionally generates high points which do not belong to any maximum in $f_S(t)$ and $f_{G2M}(t)$. For example, from the general shape of the S phase fraction curve of the population with reduced N concentration, the curve's maximum appeared to be between Hours 16 and 18 (Fig. 5). The f_S at Hour 20, which possessed a higher value, was very likely caused by random error during data analysis. If Hour 20 is taken as t_1 the duration of the terminal event is greatly underestimated, thereby increasing the estimated μ.

Results from this study and Chang & Carpenter (1988) indicate that polynomial regression is an acceptable method for smoothing phase fraction curves and revealing the locations of t_1 and t_2. However, a shortcoming of this technique occurs when peaks on phase fraction curves are very close to the starting or the end point of a 24 h sampling period. Examples can be found in the f_S and the f_{G2M} of the experiment with complete f/2 media (Fig. 2). Polynomial regression failed to generate a local maximum on either phase fraction curve. To solve this problem, the performance of other data smoothing techniques, such as fitting phase fractions with periodical functions (Slocum 1980, Keiding et al. 1984), should be evaluated. An alternative solution could be to extend the sampling period from a single 24 h cycle to 1.5 or 2 cycles. This latter approach is also helpful when the peak on f_{G2M} appears earlier than the peak on f_S during the sampling period (Fig. 2).

Growth rates estimated by the cell cycle method from 3 out of the 4 experiments gave higher values than that from cell counts. We speculate that the observed overestimation was due to the fact that the DNA method tends to overlook cell death (Chang & Carpenter 1988, Chang 1989). Since the growth rate from cell counts is the net growth rate, which takes the cell death rate into account, μ calculated from the cell cycle method should be higher. Also, dinoflagellates are known to be vulnerable to mixing (Galleron 1976, Nelson & Brand 1979, Pollingher & Zemel 1981, Karentz 1983), so it is likely that some cell death did occur in our culture of *Prorocentrum minimum*. In a previous study, the DNA

method overestimated growth rate of *Heterocapsa triquetra* by about 0.1 d^{-1} (Chang & Carpenter 1988), similar to that observed here for *P. minimum*. Regardless of whether cell death existed in the cultures, we consider this overestimation to be within the acceptable range of error for the method, especially when one considers that species-specific growth rates in the field vary well over one order of magnitude.

The difference between μ_{min} and the corresponding μ estimated from polynomial regression is smaller when the population growth rate is low. One explanation is that, according to the functional model proposed by Carpenter & Chang (1988), if the behavior of the release factor [Φ (t)] and the phase durations are kept the same, a fast-growing population will send a larger cell cohort down the cell cycle. Since a larger cohort occupies a greater width on the cell cycle, it becomes less likely for a terminal event to contain all cells in the proliferating cohort at a particular time point. In real cell populations, the fact that slow growing cells usually have longer phase durations (Olson et al. 1986; see also Table 2) further increases the possibility for the entire cohort to stay in the S+G2+M phase. This situation is also implied by the flattened depression which occurred between Hours 12 and 22 on $f_{G1}(t)$ of the experiments with reduced phosphate supply (Fig. 4). A corresponding plateau must exist on $[f_S(t)+f_{G2M}(t)]$ because the sum of $f_{G1}(t)$, $f_S(t)$, and $f_{G2M}(t)$ is always 1.

Although μ_{min} is not a good estimator for growth rate in general, it does provide a safety check for μ's estimated by other means, especially when actual growth rate is not too high. In the experiment with reduced P supply, μ_{min} was higher than μ derived from cell counts (Table 1). This indicates that a cell loss rate of 0.09 d^{-1} might actually take place in that culture. Compared to the computational procedure to estimate μ via polynomial regression, the calculation of μ_{min} is much less time-consuming.

In conclusion, the validity of the cell cycle analysis technique of measuring species-specific growth rates (Carpenter & Chang 1988, Chang & Carpenter 1988) is supported by the data in the present study. Even though *Prorocentrum minimum* had large changes in the duration of cell cycle phases, the mathematical model and DNA quantification procedures were able to calculate growth rates with acceptable error. This study supports the use of this method for measurement of dinoflagellate growth rates in batch culture. Further research is needed to assess the applicability of using the method on other phytoplankters, in particular diatoms.

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